ENVIRONMENTAL ASSESSMENT

AND

FINDING OF NO SIGNIFICANT IMPACT

ecombinant Derived Pseudorabies Vaccine -TK)

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Authorization to ship a live genetically engineered experimental pseudorabies vaccine for field safety studies under controlled conditions is granted by the United States Department of Agriculture, Animal and Plant Health Inspection Service, under the Virus-Serum-Toxin Act, to SyntroVet, Inc., for the purpose of gathering additional information in support of a license application for this biological product for use in the prevention of pseudorabies in swine.

Prepared by
Veterinary Services
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1. Introduction

I Purpose and Need

1.1 Summary

This Environmental Assessment (EA) presents and discusses scientific data and other information relevant to conducting field tests and trials out of containment with a recombinant derived live virus pseudorabies vaccine developed by SyntroVet, Inc., Lenexa, Kansas, (SyntroVet). The field tests are necessary in order to eventually satisfy vaccine safety requirements which are a prerequisite to licensing of the live virus vaccine under the Virus-Serum-Toxin Act (21 U.S.C. 151 et. seq.) by the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA). This Environmental Assessment is intended to provide the general public with documentation of the Department's review and analysis of the environmental effects that may be associated with the gathering of information in these field tests.

1.2 Field Testing of Experimental Products

1.2.1 USDA Regulations

A person may be authorized by the Deputy Administrator, Veterinary Services

(VS), APHIS, to ship experimental biological products for the purpose of evaluating them by treating limited numbers of animals. The Deputy Administrator must first determine that the conditions under which the experiment is to be conducted are adequate to prevent the spread of disease. The procedures set forth in the request for authorization to ship the experimental product must also be approved by the Deputy Administrator. Such procedures may involve special restrictions or tests when they are deemed necessary or advisable. Applicants may also be required to provide additional information in order that the Agency can assess the product's impact on the environment.

1.2.2 The Proposed Field Test

SyntroVet has submitted a request to VS to field test a genetically engineered Pseudorabies Vaccine, Modified Live Virus, S-PRV-013, under the provisions of 9 CFR 103.3. The vaccine to be tested is a live pseudorabies virus (PRV) modified by recombinant DNA techniques. The virus contains three gene deletions and one gene insertion. One deletion (tk^-) destroyed the viral thymidine kinase gene which is required for the virus to replicate in the host nervous tissue and hence cause disease. A second deletion destroyed the gene coding for a viral glycoprotein (gpX) which then prevents antibodies from being elicited to this glycoprotein. This allows vaccinated animals to be distinguished from those exposed to virus from other sources. The third deletion removed a segment of DNA from the internal and terminal repeat regions of the genome. This deletion was based on evidence with Herpes simplex virus that deletions in this region of the viral DNA produced an attenuating effect. The gene insertion consisted of a sequence coding for

biological metabolic systems, including most mammalian species. This will enable the vaccine virus to be identified in tissue cultures and has no immunological function.

Extensive safety and efficacy studies have been completed by SyntroVet in swine of all ages and in other animal species. These studies have been carried out in strict isolation, in compliance with USDA requirements, and show that the SyntroVet PRV vaccine virus:

- o is safe and effective in pigs three days of age and older
- o is safe in pregnant sows
- o is not transmitted from vaccinated animals to seronegative contact animals
- o does not replicate in central nervous system tissue and establish latent infections
- o remains genetically stable following passage in pigs
- o will not cause disease in cattle or sheep
- o induces a serologic response in vaccinated pigs that allows differentiation from infected pigs or pigs vaccinated with other vaccines

As part of the licensing requirements, the firm must conduct field trials with the candidate vaccine to evaluate performance under normal conditions of husbandry.

1.2.3 Objectives

The proposed field tests will include three studies; a bred sow/gilt study which includes 10 to 20 vaccinated animals, a piglet study involving 30 to 40

litters, and a feeder pig study which involves up to 400 vaccinated animals.

The objectives of the proposed field test are as follows:

1. To evaluate the safety of the S-PRV-013 vaccine in newborn, weaned, and breeding animals as measured by clinical observation, comparative weight gain with nonvaccinated animals, and effect on developing fetuses.

2. To attempt recovery of virus from vaccinated animals and to characterize the recovered virus as to its genetic makeup.

These field studies will be conducted in Illinois, Iowa, and Minnesota. Written permission from State regulatory authorities has been filed with USDA. The USDA authorization will be valid for 1 year from the date of issuance. A summary of the results from these trials must be filed with VS. Proposed locations in these States are as follows:

State	Proposed Sites
Illinois	Blandsville, IL Genesco, IL Gillespie, IL
Iowa	Cambridge, IA Nevada, IA
Minnesota	Adams, MN Cleveland, MN Winthrop, MN

1.3 Background: Pseudorabies

The background of Pseudorabies (Aujeszkey's Disease) has been discussed in previous environmental assessments. The most recent is the assessment regarding a PRV vaccine developed by the Upjohn Company and the Diamond Scientific Company. For more information, refer to that environmental assessment and finding of no significant impact (FONSI) dated April 29, 1987, which was announced in the Federal Register, Vol. 52, No. 86, May 5, 1987, (Upjohn PRV EA).

1.4 Purpose and Need for Recombinant Derived Pseudorabies Virus Vaccine, S-PRV-013

The purpose and need for recombinant derived Pseudorabies Vaccines have been discussed in previous environmental assesments. For specific information, see the Upjohn PRV EA mentioned above.

PRV is a herpesvirus. Herpesviruses are known to locate in nervous tissues and remain inactive there for long periods of time. These viruses may resume active infection and cause disease and shedding of the virus. The enzyme thymidine kinase (tk) is necessary for herpesvirus replication and survival. The virus must stimulate the production of the enzyme in organs such as nerve tissue which do not contain adequate levels of thymidene kinase. The deletion of the thymidene kinase gene in S-PRV-013 resulted in a virus that does not stimulate thymidine kinase and, therefore, does not reproduce in nervous

tissues. Because of the tk gene deletion, S-PRV-013 will not survive and become latent in vaccinated animals.

The gpX deletion in S-PRV-013 results in a virus that does not develop a specific glycoprotein antigen. Because of the absence of this antigen, animals vaccinated with S-PRV-013 do not develop antibodies to the glycoprotein while generating a full complement of antibody to other coat antigens. The absence of this viral antigen may be used in a test system to identify vaccinated animals that have not been exposed to wild PRV or vaccines containing the intact viral glycoprotein. This will be advantageous in eliminating disease from infected herds and in qualifying animals for interstate shipment.

The third deletion in S-PRV-013 was made in parts of the viral DNA known as the internal and terminal repeat regions. This deletion was based on studies with Herpes simplex virus showing that deletions in this area of the genome produced an attenuating effect. Results of studies with only this gene deleted from PRV confirm that the virus is less virulent in swine.

In addition to deletions discussed above, the developers of S-PRV-013 have inserted a gene for lactase as an identification marker. Laboratory tests are available which easily differentiate between the SyntroVet vaccine virus and wild virus or other currently approved pseudorabies virus vaccines. This will help distinguish this vaccine from field viruses isolated during outbreaks of disease.

II Alternatives and Description of S-PRV-013

2.1 Issuance or Non-Issuance of Authorization

The federal action in this proposal would be the issuance of a letter of authorization to permit the interstate shipment of an experimental biological product, the SyntroVet genetically engineered live pseudorabies vaccine, for the purpose of evaluating additional safety characteristics of this vaccine under restricted field conditions.

The alternative would be the non-issuance of the letter of authorization which would essentially maintain the status quo. The status quo includes the continued usage of several conventionally prepared and one recombinant DNA derived live virus vaccine. There are also field trials under way with another recombinant DNA derived pseudorabies vaccine. These are discussed in the Upjohn PRV EA referred to above.

2.2 The S-PRV-013 Experimental Vaccine

During the last 3 years, SyntroVet has been conducting research involving the genetic manipulation of the PRV genome. The research objectives were to develop a highly effective modified live virus vaccine that would have greatly enhanced safety, would elicit an antibody response that could be differentiated by simple serologic assay from responses elicited by wild-type pseudorabies virus, and could be readily identified as vaccine virus by methods easily carried out in standard diagnostic laboratories. A series of

reports describing the results of these studies and a summary is on file with USDA.

A virus construct, designated S-PRV-013, was selected for further study in animals and as a candidate Master Seed Virus. The animal studies as well as the tests conducted to certify the Master Seed Virus have been reported and filed with USDA. The collective body of data submitted to VS by SyntroVet addresses the issues of the safety of S-PRV-013 in the natural host, transmission of the virus among swine, pathobiology of S-PRV-013 in animal species other than swine, and survival of the virus under varying environmental conditions.

2.2.1 Deletion in the Internal and Terminal Repeat Regions

Based on evidence with the Herpes simplex virus regarding deletions in the internal and terminal repeat regions, SyntroVet constructed a pseudorabies virus with this deletion. Studies demonstrated that this virus was less virulent to swine than the parent strain.

2.2.2 Deletion in the Thymidene Kinase Gene

Because of their inability to reproduce in nerve cells, tk- herpes viruses are practically avirulent. The tk gene deletion was incorporated into S-PRV-013.

2.2.3 Deletion of a Glycoprotein Gene

The gene encoding for an antigenic but nonessential glycoprotein (gpX) is

known to be located in the unique short region of the viral DNA. Deletion of this gene results in a virus lacking the glycoprotein antigen. Animals exposed to this virus develop antibodies to other viral antigens but not to gpX. This deletion was made in the construction of S-PRV-013.

2.2.4 Insertion of a Positive Marker

In field outbreaks of disease, it is advantageous to have a quick assay available to distinguish field and vaccine strains of virus. For this reason, it is advantageous to have a vaccine virus engineered with a marker to provide ready identification. For a discussion of the lactase marker, see Appendix 1.

III Affected Environment

Pseudorabies is a contageous disease. As such, swine farms that are known to be infected with pseudorabies virus may be placed under quarantine by the Departments of Agriculture in the respective States. This means that movement of animals from PRV infected farms is restricted in accordance with the State regulations for quarantine of such farms. In general, swine can only be moved to another PRV quarantined farm or to slaughter.

Farm selection: Farms selected for inclusion in the pseudorabies virus vaccine safety studies are all known to be infected with PRV. Swine at each location not actively involved in the field test will be handled in a manner consistent with similar animals on that farm. The herd health program for each farm will not be altered. Pseudorabies vaccination of swine not involved in the trial will be consistent with the normal farm procedure. Therefore, they may or may not be vaccinated. In addition, all nontest pigs on a premises will be observed for any untoward effects which could result from possible spread of any vaccine virus on the farm. This will include observation for respiratory signs, CNS signs, mortality, weight loss, and other signs of illness consistent with suspected viral infection. If the test site has a large swine population and multiple building sites, principal observations will be confined to the facility in which the trial pigs are housed.

Sanitation: Routine sanitation practices will be followed at each trial

location to assist in reducing the spread of disease. These practices are common today in all large swine operations to prevent transmission of various contagious diseases. For additional information on the conduct of the test, see Section 4.7.

IV Environmental Consequences

4.1 Wild-Type Pseudorabies Virus

Information on the transmission, environmental persistence, risk to humans, and pathobiology of PRV may be found in the Upjohn PRV EA mentioned previously. Many domestic and wild animal species may be infected with PRV. The pig is the principal reservoir and other susceptible species usually die after becoming infected. In the open environment, the virus may persist for several days or even up to 2 to 3 weeks. The virus may persist in meat and meat products for long periods, but there is no public health risk from these foods as humans do not develop this disease. The virulence of PRV depends on the expression of several different genes.

4.2 Safety Studies Conducted by SyntroVet

4.2.1 Safety and Efficacy Studies in Weaned Pigs

After a preliminary evaluation of S-PRV-013 in 4 to 6-week-old pigs, a larger study was conducted to verify that this virus did not cause clinical disease in vaccinated animals. Studies were also conducted to determine if the virus could be recovered from tonsilar secretions of vaccinates. PRV-susceptible pigs were inoculated intramuscularly with either $10^{6.0}$ or $10^{4.0}$ 50 percent tissue culture infective doses ($TCID_{50}$) of virus, observed daily for 14 days, body temperatures recorded daily for 10 days, and tonsillar secretions collected daily beginning 2 days after inoculation and continuing through 10

days postinoculation. Tonsillar secretions were chosen as the primary site for measuring virus shedding since PRV commonly replicates in tonsillar tissue. Blood was collected weekly and the serum assayed for antibody to PRV. Three weeks after inoculation, the pigs were challenged with virulent PRV to measure the efficacy of S-PRV-013.

The virus caused no clinical disease, did not result in increased body temperatures following inoculation, and could not be recovered from tonsillar secretions. All pigs in this study were protected against clinical PRV when exposed to virulent virus. Nonvaccinated control pigs were susceptible to the challenge. S-PRV-013 is safe, effective, and non-shedding when given to weaned pigs.

4.2.2 Safety and Efficacy of S-PRV-013 in Piglets

Younger pigs are generally considered more susceptible to the adverse effects of PRV. However, there are many instances in which it is desirable to vaccinate newborn pigs. Studies were undertaken using three litters of 3-day-old piglets born to sows from known PRV-free herds and seronegative to PRV. In two litters, the piglets were given $10^{4.0}$ $TCID_{50}$ of S-PRV-013, leaving one or two piglets in each litter as noninoculated, contact controls. In the third litter, three pigs received $10^{6.0}$ $TCID_{50}$ of virus, four pigs received $10^{4.0}$ $TCID_{50}$ of virus, and one pig was left as an uninoculated contact control. The pigs were observed daily for 14 days, body temperatures recorded daily for 10 days, and tonsillar swabs taken daily from days 2-10 postinoculation. Blood samples were drawn weekly for determination of serum antibody to PRV. Twenty-one days after inoculation the pigs were weaned, and

7 days later they were challenged with virulent virus.

Clinical signs of PRV were not observed in any of the pigs following inoculation, although some of the pigs did show a temperature rise of 1 to 2° F for 1 to 3 days. Virus could not be recovered from tonsillar secretions of these animals. The contact control animals did not seroconvert (develop serum antibodies) to PRV following inoculation whereas all inoculated pigs did seroconvert. After challenge with virulent PRV, all of the inoculated pigs remained free of clinical signs of disease. However, the challenge control pigs as well as all of the contact control pigs developed typical central nervous system manifestations of PRV.

These studies demonstrated that S-PRV-013 is safe in pigs as young as 3 days of age and is not transmitted from inoculated to noninoculated littermates. This latter conclusion comes not only from the inability to isolate virus from tonsillar secretions of vaccinates but also from the fact that contact animals did not seroconvert during the 28-day postinoculation, prechallenge period, developed a typical primary antibody response following exposure to virulent PRV and were fully susceptible to the clinical effects of the challenge virus.

4.2.3. Safety of S-PRV-013 in Pregnant Sows

Virulent strains of PRV are capable of crossing the placenta in pregnant sows, resulting in abortion, fetal abnormalities and possible infertility of the sow at the next breeding. To determine if S-PRV-013 was sufficiently attenuated to be used as a vaccine virus in pregnant sows and gilts, the virus was inoculated intramuscularly at a concentration of $10^{6.0}$ $TCID_{50}$ into two

seronegative sows at approximate 45 days gestation. The sows were monitored daily and body temperatures recorded. Five weeks post-inoculation, both sows were sacrificed and their entire uterus collected. Fetal tissues were assayed for the presence of virus and sera were collected to determine PRV antibody levels.

Neither sow developed clinical disease associated with PRV. One sow developed a bacterial infection (Hemophilus) 3-weeks postinoculation but recovered following antibiotic therapy. As a result of this bacterial infection, five of the 15 fetuses recovered from this sow were dead and decomposed. The other fetuses were normal in appearance, and all fetuses from the second sow were normal. Virus could not be isolated from samples of tonsil, lymph nodes, brain, liver, lung or spleen of any of the fetuses. None of the fetal sera had PRV antibodies. However, serum from both sows had PRV antibody.

A study was undertaken to determine the safety of S-PRV-013 following intravenous injection into seronegative pregnant sows. Three sows at approximately 45 days gestation were obtained from a known PRV-free herd. One sow was inoculated with $10^{6.3}$ $TCID_{50}$ of virus, a second sow was inoculated with $10^{4.3}$ $TCID_{50}$ virus, and a third sow was given uninfected cell culture fluid as a negative control. The animals were observed for evidence of clinical disease daily through 14 days and every other day through 35 days postinoculation. Temperatures were recorded daily for 14 days. At 5 weeks postinoculation all three sows were sacrificed and their entire uterus collected. Virus isolation was attempted from both fetal and maternal tissue of all three sows. Antibodies to PRV were evaluated in fetal and sow sera.

All three sows remained clinically normal throughout the observation period, and all fetuses taken from these sows were normal in appearance at the time of sacrifice. Attempts to isolate virus from maternal blood or from tonsil, lymph node, brain, liver, lung or spleen tissues taken from the fetuses were negative. The fetuses had no detectable PRV antibody, whereas both sows given S-PRV-013 did seroconvert after inoculation. The negative control sow did not develop PRV antibody. In this study, the opportunity for the virus to cross the placenta and infect fetal tissue was much greater because of the intravenous route of delivery. There was no evidence that this had occurred. This test demonstrates that the virus is safe for use in pregnant sows.

The most sensitive PRV swine host is the developing fetus. A study was conducted to assess the effects of S-PRV-013 following direct inoculation into fetuses in utero. Laparotomies were performed on two sows of approximate 80-days gestation. Fetuses in the right uterine horn of one sow were inoculated with $10^{2.0}$ $TCID_{50}$ of virus, and fetuses in the left uterine horn were not inoculated. Fetuses in the right uterine horn of the other sow were inoculated with uninfected cell culture lysate, and fetuses in the left uterine horn were not inoculated. The sows were observed daily and body temperatures recorded. Fourteen days after inoculation, the sows were sacrificed and the uterus collected. Tissue was collected for virus isolation, and blood was collected for determination of serum antibody to PRV.

All fetuses taken from the sow inoculated with the cell culture lysate were normal in appearance and did not yield virus when samples were cultured. All of the fetuses inoculated with virus were dead, and three of seven fetuses from the uninoculated horn were autolyzed. Virus was recovered from the

tonsil of one inoculated fetus and from the lung/spleen of three inoculated fetuses. Four of seven amniotic fluid samples and five of seven placental tissues from the right uterine horn were positive for virus. Virus was not recovered from any of the samples taken from the fetuses or placenta of the uninoculated uterine horn.

Whereas this virus retains virulence in the developing fetus, it has a limited ability to replicate since it did not spread to the uninoculated uterine horn or cause abortion.

4.2.4 Safety of S-PRV-013 in Other Species

It is not uncommon to observe clinical PRV and death in cattle, sheep, cats, and dogs on swine farms where pseudorabies is known to be endemic. The virulence of S-PRV-013 was assessed in these four animal species.

Considering that PRV is shed from infected swine via the oro-nasal route but the mode of transmission from infected swine to other animal species can be varied, a concentration of $10^{7.0}$ plaque forming units (PFU) of S-PRV-013 was inoculated by intramuscular injection and by instillation into the nasal passages and into the throat.

Three 5 to 6-month-old calves, three 5 to 6-month-old lambs, and five 4 to 5-month-old kittens were each inoculated with $10^{7.0}$ PFU of S-PRV-013 by the three routes listed above. The animals were observed daily for 28 days, and body temperatures were recorded daily for 14 days. Blood samples were taken at weekly intervals to determine serum antibody titers to PRV.

All of these animals remained normal throughout the observation period and none exhibited a febrile response. Twenty-eight days postinoculation, one of three calves had a PRV antibody titer of 1:2, the three lambs had antibody titers of 1:4 and 1:8, and three of five kittens had 1:2 antibody titers. The absence of virulence for these animal species is significantly different from what is reported for wild type virus and the BUK vaccine strain especially considering the high concentration of virus given.

A group of five 5 to 6-month-old pups were inoculated with 10^6 PFU of S-PRV-013 and observed as above. Approximately 5-days postinoculation, two dogs showed hind leg paralysis, and by day 6, the other three dogs were exhibiting similar signs. Pruritis developed at the injection site, and two of the dogs became comatose. All dogs were euthanized, and tissue samples were taken for further evaluation. Virus was recovered from brain tissue of all five dogs, and the sciatic nerve of all five dogs reacted positively with fluorescein conjugated pseudorabies antiserum.

These studies were then extended to determine a comparative LD_{50} of S-PRV-013 and its parent Iowa strain virus. The Iowa strain was inoculated into 2 pups each at concentrations of $10^{2.78}$ PFU or $10^{4.78}$ PFU. S-PRV-013 was inoculated into two pups each at concentrations of $10^{2.95}$ and $10^{4.95}$ PFU. All four dogs inoculated with the Iowa strain developed pruritis, hind leg paralysis, and became comatose before being euthanized. None of the four dogs inoculated with SPRV-013 showed clinical signs throughout 28 days observation. The results of these two studies demonstrate that although the S-PRV-013 virus retains some virulence for dogs, it is reduced by at least 150 fold from its

parent virus.

These studies show that S-PRV-013 is no longer virulent or has greatly reduced virulence for other animal species known to be infected by PRV.

4.3 Persistence of S-PRV-013 in the Environment

SyntroVet conducted a number of experiments to determine survivability of S-PRV-013 outside of the animal body. In summary, these data show that the virus has not acquired an increased ability to survive under various environmental conditions. S-PRV-013 is slightly more fragile under the test conditions used, and it can be predicted that the survival of S-PRV-013 in the environment will be less than that normally found with field virus. The results of these studies are presented in Appendix 2.

4.4 Shedding Patterns of S-PRV-013 in Swine
Fate of the Virus and Recrudescence Studies

The ability of a virus to persist in its natural host or to cause abnormal clinical disease is measured by defining the target tissues for viral replication and by determining the length of time such virus can be recovered from these tissues. Following natural infection with virulent virus, the primary site of viral replication is the upper respiratory tract. Virus can be isolated early from tonsils and olfactory epithelium and shortly thereafter from the medulla and pons. Virus is also found in the cranial nerves and ganglia. Secondarily, the virus can be isolated from liver, spleen, kidney,

and adrenal glands. Viremia is of a low level and difficult to detect.

Following intranasal inoculation of the Iowa strain into weaned pigs, virus has been demonstrated in multiple tissues for 6-days postinoculation. The frequency of isolation from multiple sites declined with time. However, virus was still recoverable from tonsil and trigeminal ganglion at 2, 6, and 13 months postinoculation.

To follow persistence of S-PRV-013 following inoculation of susceptible animals, four litters of 3-day-old piglets were inoculated intramuscularly with $10^{6.06}$ $TCID_{50}$ of virus. Tonsillar swabs were taken daily from Days 2-10 postinoculation. On Days 2, 4, 6, 8, and 10 postinoculation, one piglet from each litter was sacrificed and tissues removed for virus isolation. All remaining piglets were weaned 21 days after inoculation and 7 days later, the pigs were treated with steroid (Dexamethasone, 2 mg/Kg/day for 7 days) in an attempt to recrudesce virus that may have become latent in central nervous system tissue. Virus isolation attempts (presteroid and poststeroid treatment) were made from tonsillar swabs, and homogenates of brain, lymph nodes, and a mixed lung, liver, and spleen. Additional samples of brain and of trigeminal ganglion were cocultivated with Vero cells and observed for cytopathology for 30 days before being considered negative.

Virus was recovered from five tonsillar swab samples prior to steroid treatment, but after steroid treatment virus was not recovered from any of the tonsillar swabs. Only one pig in each group of four pigs sacrificed on days 2, 4, 6, and 8 postinoculation had recoverable virus in the tissues examined. Virus was not recovered from the four pigs sacrificed on Day 10. No virus

could be recovered from any of the 23 pigs (tonsillar swabs or tissues) given steroid and sacrificed 7 and 14 days later. Cocultivation of brain and trigeminal ganglion tissues, taken presteroid and poststeroid treatment, were also negative.

Of the positive swab and tissues samples, seven occurred during the first 4 days of the study and no isolates were made after day 9 of the study. These data clearly demonstrate that unlike wild PRV and the parent Iowa strain, S-PRV-013 only persists in inoculated pigs for a short period. The most striking difference between S-PRV-013 and the parent strain is the inability to recover S-PRV-013 from central nervous system tissue or to demonstrate latent virus.

4.5 Reversion of Recombinant Vaccine to Virulence

Back Passage Studies with S-PRV-013 in Weaned and Newborn pigs

A primary safety consideration in the development of any live virus vaccine is whether the vaccine virus sheds from vaccinated animals, and if it does, what the consequences of that shedding may be. Any live virus that replicates in a vaccinated animal has the potential to be shed from that animal. Whereas shedding of vaccine virus may contribute to "herd immunity" (nonvaccinated animals become immune as a result of being exposed to vaccinated animals shedding the vaccine virus), a potential negative consequence of the shed and spread of vaccine virus is the possibility for the virus to revert to a virulent form following several such passages through its natural host.

Weaned Pigs. SyntroVet's initial studies were designed to back passage the virus by intranasal inoculation of susceptible pigs of weaning age. In the first experiment, five 4-week-old pigs were each inoculated intranasally with $10^{5.7}$ $TCID_{50}$ of S-PRV-013 virus. Nasal and tonsillar swabs were collected daily from days 2-14 following inoculation and the pigs were observed daily for clinical signs of PRV. All of the pigs remained clinically normal and did not exhibit a febrile response. All pigs seroconverted to PRV but virus could not be recovered from any of the swab samples.

A second study was undertaken using 3-week-old pigs and instilling the same amount of virus as before, only this time it was delivered into the nostrils and into the back of the throat to ensure that virus reached the tonsilar area. These three pigs remained clinically normal and afebrile during the 14-day observation period. Virus was recovered for one day (day 2) from one of the three inoculated pigs. This virus was designated as pig passage number one. Because of the apparent low level of replication of this virus in the nasal passages and tonsillar area of 3 to 4-week-old pigs, the attempts to continue back passage of this virus were done in newborn pigs.

Newborn Pigs. A litter of pigs born to a PRV-seronegative sow was inoculated at 2 days of age by instilling $10^{5.76}$ PFU of pig passage No. 1 virus nasally and orally into six of eight pigs in the litter. The remaining 2 pigs were left as uninoculated contact controls. The pigs were observed daily, body temperatures recorded daily, and nasal and tonsillar swabs taken daily from 2 to 14 days postinoculation. All six inoculated pigs and one contact control developed body temperature increases of 1° to 2° F for 2 to 7 days. No clinical signs were observed in any of the pigs, and all pigs exhibited a

normal appetite and weight gain. Virus was recovered from all six inoculated pigs and from one of the contact control pigs (No. 8). All eight pigs in the litter developed PRV antibody. Virus from pig No. 8 was used for further passage. This virus was considered to be pig passage No. 3, passage No. 2 occurring when the virus was shed from an inoculated pig to pig No. 8.

A second litter of 2-day-old pigs from a PRV-negative sow was used for the next back passage. Five piglets were each inoculated orally and nasally with $10^{5.5}$ PFU of virus from pig No. 8. The other two pigs in the litter were left as uninoculated contact controls. The study was carried out as described above. Three of five inoculated piglets and one of two contact piglets developed a febrile response of 1 to 2-days duration. There were no clinical signs of disease in any of the pigs. Virus was recovered from only four of the inoculated pigs and for a much shorter time than in the first litter. Virus was not recovered from either of the contact control animals. All five inoculated pigs developed low serum antibody titers but neither of the contact control animals seroconverted. Virus recovered from the pigs in this study was considered to be pig passage No. 4.

The passage No. 4 virus was subjected to a genetic analysis to determine if its genotype had remained stable through these passages in the pig. A plaque assay of the passage No. 4 virus and application of the BLUOGAL test to the viral plaques demonstrated that 100 percent of the plaques contained virus with the lactase protein. Extraction of the viral DNA and digestion with restriction enzymes gave gel patterns identical to those seen with the input S-PRV-013 Master Seed Virus.

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In summary, attempts to back passage the S-PRV-013 virus through pigs and to determine if reversion to virulence or reversion of any of its genetic characteristics had occurred have shown the virus to be stable and to lose ability to replicate following passage. The results presented demonstrate that S-PRV-013 will not be transmitted to nonvaccinated pigs when administered according to normal practice. Also, the virus does not have the ability to continue to replicate.

For a discussion prepared by SyntroVet concerning potential in vivo recombination, see Appendix 3. Studies with virus containing the intact gene for thymidene kinase and with the gene for lactase replacing the gene for gpX were less virulent in swine than wild type virus.

4.6 Oncogenicity

Wild type PRV found in nature is not a tumor virus. There have been no reports of oncogenicity associated with PRV infection in any animal species. The recombinant DNA methods used for the preparation of S-PRV-301 do not introduce any new information into the already nontumourigenic PRV virus which could cause it to become tumourigenic.

4.7 Monitoring of Test Animals

A monitoring program has been established as follows:

Observation period: All test animals will be observed for 35 days following

vaccination for any adverse reactions.

Disposition of trial animals: At the completion of the clinical phase of the trial, the test animals will be handled in a fashion similar to that of other farm pigs at the same stage of the production cycle. The sows and gilts will be returned to the breeding herd unless culled, and feeder pigs will remain with other on-farm feeder pigs and eventually be sent to slaughter when they reach market weight. In either case, these farms are quarantined and animal movement is restricted according to the terms of the quarantine.

Proximity to other hog producing areas: The test farms will generally be in PRV endemic areas. They may be under State quarantine. Contact and interaction with other local production units will be restricted.

Proximity to large population centers: Farms selected for these safety field trials will not be close to large human populations centers.

Restriction of Movement: Test animals cannot be moved from a trial site to a non-PRV quarantined farm. These animals may move only to another PRV quarantined farm or to slaughter.

Disposition of test animals if they become infected with PRV: If test animals on any of the trial sites become infected with PRV, they will be handled consistently with the terms of the quarantine.

V Consultation and Coordination with other Agencies, Organizations and Persons

The following people and their affiliations were involved in preparing the document and reaching and verifying the proposed action.

1. State approval (See Appendix 4)
2. Veterinary Biologics Staff Reviewers - Dr. G. Shibley and Dr. R. Miller
3. Chief Staff Veterinarian, Biologics Staff - Dr. D. Espeseth
4. Biotechnology & Environmental Coordination Staff - Mr. T. Medley, Esq., and Mr. M. Werner, Esq.
5. Veterinary Services Biotechnology Committee -

   Dr. B. Johnson (Chairman), VS, APHIS

   Dr. P. Joseph, VS, APHIS

   Dr. S. Wilson, VS, APHIS

   Dr. D. Espeseth, VS, APHIS

   Dr. B. Nervig, National Veterinary Services Laboratory, VS, APHIS

   Dr. D. Berkowitz, Food Safety Inspection Service, APHIS

   Dr. J. Miksche, Agriculture Research Service, Science & Education

   Dr. W. Gartland, Office Recombinant DNA Activities, National Institute of Health, Health & Human Services

   Dr. H. Miller, Food & Drug Administration, Health & Human Services

   Mr. A. Samofal, Esq., Office of General Counsel, USDA

VI Finding of No Significant Impact

APHIS has found that its action in approving the proposed field studies would not have a significant impact on the environment. Such findings are supported by the following facts:

1. In the development of S-PRV-013, genetic engineering procedures were employed to facilitate three gene deletions and one gene insertion in the pseudorabies virus genome. One deletion destroyed the viral thymidine kinase (tk) gene which is required for the virus to replicate in the host's nervous system and hence cause disease. A second deletion destroyed a gene coding for a viral glycoprotein (gpX) which then prevents antibodies from being elicited to this glycoprotein, thus allowing vaccinated animals to be distinguished from those infected with a field virus. The third deletion resulted in a nonspecific reduction in virulence. The gene insertion codes for an enzyme, lactase, commonly found in nature and mammalian cells. The presence of this gene does not increase virulence or the ability of the virus to survive in the environment.

2. The S-PRV-013 vaccine virus was shown to be avirulent and yet fully capable of eliciting an immune response that protects pigs from PRV. It did not elicit antibodies to gpX which allows for differentiation between infected pigs and vaccinates. The addition of the lactase gene confers no increase in the virulence or persistence of the virus, but serves to distinguish between S-PRV-013 and wild or other vaccine viruses.

3. Transmission of S-PRV-013 could not be demonstrated from inoculated pigs.

In one study, vaccine virus was detected on tonsil swabs taken from five of 43 vaccinated pigs. Attempts to recover latent virus from these pigs were unsuccessful. In another study, anti-PRV antibody could not be detected in a serum neutralization assay using serum taken from sentinel pigs after 21 days of contact with vaccinates. Sentinel animals remained fully susceptible to challenge with wild-type PRV. In backpassage studies, spread from nasally administered vaccine was reported once in 2-day-old swine. The ability of the virus to spread and reactions to the virus declined with each passage. The virus did not revert to virulence in these studies.

4. The tk gene deletion is a stable characteristic of the vaccine virus with a probability of reversion being essentially zero. The lactase gene insertion likewise remains stable through repeated passes in culture and in swine.

5. The wild-type pseudorabies virus is found widely distributed in nature, and it does not contain an oncogene or cancer-causing substance. The insertion of the commonly distributed lactase gene does not contain oncogenes and the product, lactase is not oncogenic. There is no likelyhood that S-PRV-013 is oncogenic.

6. The wild-type pseudorabies virus is not considered pathogenic to man. The recombinant vaccine differs from wild-type pseudorabies by deletions and insertion of an inocuous gene, and also is considered nonpathogenic in man.

This EA and FONSI have been prepared in accordance with (1) the National Environmental Policy Act of 1969 (NEPA) (42 U.S.C. 4331 et. seq.); (2) Regulations of the Council on Environmental Quality for Implementing the

Procedural Provisions of NEPA (Title 40, Code of Federal Regulations (CFR) Parts 1500-1508); (3) USDA regulations implementing NEPA (7 CFR Part 1b); and (4) APHIS Guidelines Implementing NEPA (44 FR 50381-50384 and 44 FR 51272-51274).

This document presents an in depth discussion of scientific data and other information which was considered by APHIS in its decision concerning authorization to ship the SyntroVet recombinant derived live pseudorabies virus experimental product (S-PRV-013) for field testing under restricted conditions. The Environmental Assessment describes the disease, the causative agent, conventional methods of treatment, and the recombinant derived virus and vaccine produced from it. The APHIS alternative would have been to refuse to allow shipment for purposes of the field test. This action would result in preventing the vaccine developer from conducting the necessary studies required to satisfy safety standards. This would in effect prevent the licensing of the vaccine and deny its sale in the marketplace. Therefore, farmers would be denied a vaccine which contains a serologic marker to differentiate vaccinated animals from those infected with the wild-type virus.

Finally, APHIS prepared an Environmental Assessment and Finding of No Significant Impact on April 21, 1986, regarding a similar vaccine, i.e., TechAmerica's recombinant derived thymidine kinase negative (tk^-) pseudorabies virus vaccine, modified live viurs. On April 29, 1987, APHIS prepared an Environmental Assessment and Finding of No Significant Impact in support of field trials with a similar genetically engineered vaccine developed by the Upjohn Company and Diamond Scientific Company. This genetically engineered

experimental vaccine (S-PRV-013) has essentially the same safety characteristics as those already approved.

Appendix 1

Properties and Distribution of Lactase

The Enzyme Committee of the International Union of Biochemistry classify enzymes on the basis of their function and their enzyme activity, (see "Enzyme Nomenclature", The International Union of Biochemistry, Elsevier Publishing Co., 1965). The enzyme beta-galactosidase is given the number EC-3.2.1.23 as its unique identifier. The common name for this enzyme is "lactase".

The source of the lactase gene inserted into S-PRV-013 IS Escherichia coli. E. coli is an ubiquitous microorganism found in the intestinal tract of animals. Some strains are pathogenic, causing gastric upset and diarrhea. Other strains do not cause disease. All animals (except those raised germ-free) are exposed to E. coli and to E. coli lactase. The company reports having analyzed many samples of serum from animals and never finding one that did not contain antibodies to E. coli lactase. Thus the animal had been exposed to E. coli and this resulted in direct exposure of the immune system to lactase followed by mounting an immune response as they would to any other antigen.

It should be noted that the ONLY gene from E. coli present in Syntro's vaccine is the lactase gene. Any animal given the vaccine will have already seen this lactase previously, or (if very young) would see it soon due to natural exposure to E. coli.

Lactase splits the sugar molecule lactose (a disaccharide) into two parts, glucose and galactose. This is the first step in the pathway for the utilization of lactose in energy metabolism in the cell. Lactose is the sugar molecule present in milk. Every mammal has a gene that codes for lactase that enables the young animal to digest milk sugar. In most mammals, this gene is active during the early life when milk is the sole source of nourishment, and the enzyme is released into the gut to digest lactose. A lack of the enzyme

lactase leads to a lactose intolerance syndrome in humans. Infants with this syndrome must be fed a soy based formula that contains no lactose.

In most mammals, the gene for lactase is turned off at weaning, but in some human ethnic groups, the gene is active throughout life allowing these people to continue to drink milk throughout adulthood. Ethnic groups that do not have the lactase gene in adulthood may eat and drink a fermented milk product (yoghurt or similar product). In this case, the microorganism responsible for fermentation has supplied the lactase.

A number of microorganisms (but not all) produce lactase. Microorganisms that contain the enzyme are able to utilize lactose as the sole carbon source. The ability of an organism to utilize lactose does not correlate with the pathogenicity of the organism; for example the pathogenic Salmonella and Shigella do not metabolize lactose, while E. coli does. Similarly, nonpathogenic microorganisms may or may not have lactase.

The dairy industry has exploited microbial lactase in the production of milk products. The following is a list of the microorganisms used in the fermentation of various products:

Buttermilk . . Lactobacillus bulgaricus

Yoghurt. . . . Lactobacillus bulgaricus + Streptococcus thermophilus

Kefir. . . . . Streptococcus lactis + L. bulgaricus + yeasts

Cheeses. . . . S. lactis, + many others

One of the ingredients contributed to the process by the microorganism is the enzyme lactase which breaks down the lactose sugar and makes these dairy products digestible for human adults. The microorganism plus the lactase remain in the food and are eaten (from "The Microbial World", R.Y. Stanier et. al., Prentice-Hall, 1976).

The history of the use of lactase in the food industry illustrates the safety associated with this enzyme in the human food chain. The results of studies reported by the firm confirm the safety of this enzyme in the environment and demonstrate no negative consequences from inserting the lactase gene into S-PRV-013.

The lactase enzyme is present throughout the animal kingdom and exhibits biologic activity when its substrate, lactose, is present. In the absence of a substrate, or after the enzyme has performed its function, the lactase protein, like any other protein, will be degraded by proteolytic enzymes. Introduction of lactase into mammalian cells with S-PRV-013 vaccine will ultimately result in degradation of the enzyme with no unusual effects.

In field outbreaks of disease, it is advantageous to have a quick assay available to confirm or eliminate the possibility of vaccinal organisms contributing to the outbreak. SyntroVet developed a vaccine virus engineered with a marker to provide ready identification.

The features of this positive marker are that it is a protein commonly found in the environment and that it allows rapid identification of the vaccine virus using conventional laboratory tests. The protein selected for insertion is lactase (beta-galactosidase), an enzyme found in animals and humans, whose function is to digest the sugar, lactose, into two simpler sugars, glucose and galactose. This enzyme can readily be identified by use of a commercially available test. As applied to S-PRV-013, the Bluogal ™ indicator is added to plates of a typical PRV plaque assay. Plaques arising from a virus carrying and expressing the lactase gene will turn blue on addition of the indicator.

Appendix 2

COMPARATIVE SENSITIVITY OF IOWA STRAIN AND S-PRV-013 TO VARIOUS ENVIRONMENTAL CONDITIONS

The purpose of these experiments was to determine if the genetically modified virus has acquired increased ability to survive adverse conditions and thus to persist for longer periods of time in the environment. The parental Iowa strain PRV and S-PRV-013 were subjected to various conditions that would mimic natural environments in which pseudorabies viruses might exist. After various periods of exposure the viruses were assayed for residual infectivity. The results of these studies are presented in Tables 1, 2 and 3.

Each of the viruses was placed in buffered solutions at pH 3.0, 5.0, 7.0 and 9.0. Samples were withdrawn immediately and after 2 hours or 4 hours exposure at ambient temperature and assayed for residual infectivity (Table 1). Both viruses were inactivated immediately by exposure to pH 3.0. At pH 5.0, 7.0 and 9.0, the Iowa strain virus lost very little infectivity over the 4 hour sampling period. The survival of PRV-013 at pH 5.0 and at pH 9.0 was less than observed for Iowa strain.

Each virus was placed in nonchlorinated water, held at room temperature and sampled at 0, 3, 6, 24, and 72 hours. Both viruses lost less than 1.0 log10 infectious virus during the 72 hour period, however, the loss occurred more quickly (3 hours) with PRV-013 (Table 2).

A cornmeal-milk slurry was prepared and each virus was placed in this slurry and held at room temperature or at 37° C and sampled at 0, 3, 6, 24, and 72 hours. The Iowa strain virus was stable during the first 6 hours at room temperature whereas the infectivity titer of PRV-013 had dropped by 1.13 log10 at 6 hours (Table 2). By 24 hours both viruses had lost at least 3.88 log10 infectious units. Following incubation at 37° C, the infectivity of both

viruses had dropped by over 1 log10 after 3 hours. After 6 hours the loss of infectious Iowa strain virus was 1.9 log10, but it was 2.38 log10 for PRV-013. Each of the viruses was placed in a Petri dish containing nonchlorinated sterile water approximately 2-3 mm deep and exposed to ultraviolet light. The Iowa strain virus did not lose detectable infectious virus after two minutes exposure, but after 10 minutes exposure, approximately 4.9 log10 infectious virus was lost (Table 3). The ability of PRV-013 virus to survive UV exposure was less 2.88 log10 of infectious virus, was lost after only 2 minutes exposure, and greater than 4.25 log10 loss was recorded after 10 minutes exposure.

In summary, these data show:

S-PRV-013 MSV has not acquired an increased ability to survive under various environmental conditions. S-PRV-013 is slightly more fragile under the test conditions used. It could be predicted that the survival of S-PRV-013 in the environment will be less than that normally found with field virus.

TABLE 1

A COMPARISON OF THE ENVIRONMENTAL SURVIVAL OF S-PRV-013 AND IOWA PRV

Environmental Condition	Virus	Titer[a] Following Exposure 0 Hr	2 Hr	4 Hr	Infectivity Loss
pH 3.0 Ambient Temperature	Iowa	3.5	1.5	1.5	--
	S-PRV-013	3.5	1.5	1.5	--
pH 5.0 Ambient Temperature	Iowa	6.0	6.0	>5.5	0.5[a](68.4)[b]
	S-PRV-013	4.88	4.25	4.13	0.75(82.2)
pH 7.0 Ambient Temperature	Iowa	7.0	7.0	7.0	0(0)
	S-PRV-013	5.5	5.13	5.38	0.12(24)
pH 9.0 Ambient Temperature	Iowa	7.25	7.0	7.13	0.12(24)
	S-PRV-013	5.5	5.38	4.75	0.75(82.2)

[a] Log10 TCID50/ml

[b] Percent loss of infectious virus

TABLE 2

A COMPARISON OF THE ENVIRONMENTAL SURVIVAL OF S-PRV-013 AND IOWA PRV

Environmental Condition	Virus	Titer[a] Following Exposure For 0 Hr	3 Hrs	6 Hrs	24 Hrs	72 Hrs	Infectivity Loss
Non-Chlorinated Water Ambient Temperature	Iowa	7.13	7.0	6.75	7.13	6.25	0.88[a](87)[b]
	S-PRV-013	6.0	5.5	5.63	5.63	5.38	0.62(76)
Cornmeal-Milk Slurry Ambient Temperature	Iowa	6.63	6.38	>6.5	2.75	1.5	>5.13(>99.99)
	S-PRV-013	6.38	5.63	5.25	2.5	1.5	>4.88(>99.87)
Cornmeal-Milk Slurry 37° C	Iowa	6.63	5.5	4.75	--b	--b	1.88(98.7)
	S-PRV-013	5.88	4.63	3.5	--b	--b	2.38(99.6)

[a] Log10 TCID50/ml

[b] Percent loss of infectious virus

[c] Samples contaminated with bacteria

TABLE 3

A COMPARISON OF THE ENVIRONMENTAL SURVIVAL OF S-PRV-013 AND IOWA PRV

Environmental Condition	Virus	Titer[a] Following Exposure 0 min	2 min	10 min	Infectivity Loss[b]
Ultraviolet Light	Iowa	6.5	6.38	1.63	>4.87(>99.87)
Ambient Temperature	S-PRV-013	5.88	3.0	1.63	>4.25(>99.44)

[a] Log10 TCID50/ml

[b] Percent loss of infectious virus

Appendix 3

ABILITY OF S-PRV-013 VIRUS TO RECOMBINE WITH OTHER GENETICALLY MODIFIED PRV STRAINS

When cells or animals are infected simultaneously with two strains of the same microorganism, each defective in a different property, the possibility exists that restoration of the defective property may occur. This phenotypic expression may have different genotypic explanations. It may be that the defective gene product was supplied by each complementing strain without any change in the DNA, thereby leaving the original infecting strains intact.

Alternatively, there could have been a recombinational event occur at the DNA level, restoring the defective gene with a fully functional gene. It is recognized that this occurs in nature, and it can occur under laboratory conditions with a much higher frequency than occurs naturally. In fact, capitalizing on this basic knowledge of genetic recombination has been the basis of genetic engineering technology.

An issue of interest is the possibility that two gene-deleted vaccine strains might recombine in the host, resulting in a virus with partial or complete virulence. This possibility, of course, also exists with any of the current modified live virus vaccines and wild type strains of the virus. With specific regard to S-PRV-013, or other gene-deleted PRV vaccine viruses, that also carry a diagnostic gene deletion, an additional consequence of recombination is the possibility that the recombined virus might still carry the diagnostic deletion, yet be capable of causing disease in the host.

Because S-PRV-013 has been engineered with three deletions, the possibility of restoring all of these deletions at any one time is extremely remote. Studies have been designed to test the hypothesis of in vivo recombination and this

will also be a subject of certain planned field studies. Additionally, a series of laboratory tests is available that can readily be used to determine if any one or more of these deletions has been restored in virus isolated from infected animals.

Appendix 4

Letters of Authorization from State Authorities

STATE OF MINNESOTA
BOARD OF ANIMAL HEALTH
160 AGRICULTURE BLDG.
90 W. PLATO BLVD.
ST. PAUL, MN 55107
(612) 296-2942

JUN 05 1987

SyntroVet

June 2, 1987

Dr. Janis K. McMillen
Vice President
Product Development & Registration
SyntroVet Incorporated
9669 Lackman Road
Lenexa, Kansas 66219

Dear Dr. McMillen:

This letter is in response to your request of May 22, 1987, for permission to conduct safety field trials in swine herds with SyntroVet's live recombinant pseudorabies vaccine.

Approval is hereby granted to conduct the safety trials as described in the Field Trial protocal and in accordance with CFR, Title 9, Chapter 1, Part 103, Section 103.3.

In addition you must provide us with the names of the participating veterinarians and the location of the herds.

Sincerely,

T. J. Hagerty, D.V.M.
Executive Secretary

TJH:msc

IOWA DEPARTMENT OF AGRICULTURE

DIVISION OF ANIMAL INDUSTRY
Wallace Building
DES MOINES, IOWA 50319

JUL 0 2 1987

SyntroVet

SyntroVet Incorporated

DATE June 26, 1987

Dr. W. D. Felker, State Veterinarian

SUBJECT Your Request

Permission is hereby granted to conduct safety field trials in swine herds in Iowa with your live recombinant pseudorabies virus vaccine. All studies and shipment of vaccine will be compliance with requirements of 9CFR 103.3, and that detailed records will be maintained by each investigator.

W. D. Felker DVM

0638 1/76

State of Illinois
DEPARTMENT OF AGRICULTURE
Division of Meat, Poultry and Livestock Inspection
State Fairgrounds / P.O. Box 19281 / Springfield 62794-9281

Administrative Unit (217) 782-4944
Bureau of Animal Health (217) 782-4944
Bureau of Animal Welfare (217) 782-6657
Bureau of Meat and Poultry Inspection (217) 782-6684

May 26, 1987

Dr. Janis K. McMillen
Vice President
Product Development & Registration
SyntroVet Incorporated
9669 Lackman Road
Lenexa, Kansas 66219

Dear Dr. McMillen:

This is to grant permission for SyntroVet Incorporated to conduct safety field trials on SyntroVet's live recombinant pseudorabies virus vaccine in Illinois. This permit in no way involves the Illinois Department of Agriculture or me with any liability relative to the use of this product in Illinois.

Very truly yours,

DIVISION OF MEAT, POULTRY
AND LIVESTOCK INSPECTION

Paul B. Doby, D.V.M.
Superintendent

kf